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Regulation of the transcription factor, CTCF, by phosphorylation with protein kinase CK2

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Abstract CTCF is a transcription factor involved in various aspects of gene regulation. We previously reported that CTCF function is modulated by protein kinase CK2. In this report we investigate further the role of CK2 in regulating the transcriptional properties of CTCF. We demonstrate that coexpression of CTCF with CK2 switches function of CTCF from repressor to activator. The non-phosphorylatable mutant increases repression by CTCF and potentiates the growth-suppressive ability of the protein, whereas the phospho-mimetic mutant behaves in the opposite fashion. Mutation of the individual serines reveals that Serine 612 is a critical residue in regulation of CTCF by CK2.

Keywords: CTCF; Protein kinase CK2; c-myc; Phosphorylation; Transcription

1. Introduction

CTCF is a highly conserved eleven zinc-finger (ZF) transcription factor that has multiple DNA-binding sequence specificity; its target sites have been characterised in the promoters, silencers, insulators, and other regulatory regions of several genes [1–7]. Depending on the promoter context and cell background, CTCF may repress [3,8–10] or activate [11] transcription, however, its repression function predominates. CTCF plays the paramount role in genomic imprinting [12,13] and is involved in X-chromosome inactivation in mammals [14].

CTCF is a nuclear protein ubiquitously expressed in many different proliferating and differentiated cell types [2,3]. The presence of the CTCF protein in many cell types raises the possibility that post-translational modification could be a mechanism controlling transcriptional properties of the universally expressed CTCF protein. Our findings revealed that phosphorylation by CK2 [15] and poly(ADP-ribosyl)ation [16] are the mechanisms regulating the activity of CTCF post-translationally. Phosphorylation profile of CTCF was found to be dynamic depending on a given cell stage/line differentiation pathway [17]. A growing body of evidence also demonstrate that CTCF may be involved in the TGFβ signalling pathways [18,19].

Phosphorylation appears to be one the most important and most studied form of post-translational modifications involved in the regulation of transcription factor providing a link between signal transduction and expression of genes [20–22]. Phosphorylation controls the function of transcription factors at different levels. It can affect, either positively or negatively, DNA binding affinity, trans-activating/-repressing function or compartmentalisation of transcription factors [23,24]. Among the protein kinases, protein kinase CK2 (CK2), or former casein kinase II, is known for phosphorylation of proteins involved in regulation of transcription, signalling, proliferation, various steps of development, metabolic processes and also DNA repair [22,25,26]. CK2 is highly conserved in evolution and has been found in all eukaryotic cells investigated so far [25]. CK2 is a dynamic molecular complex composed of two subunits a and a′ (representing the catalytic domain of CK2) and a dimer of the β-subunits (representing the regulatory domain of CK2) [27,28]. It phosphorylates serines and threonines immersed in acidic sequences within proteins and peptides and the minimum requirement for phosphorylation is the sequence S∗/T∗XXDE (in which asterisk denotes the phosphorylated serine or threonine and X represents any non-basic amino acid) [22]. In addition, the residue in the third position towards the carboxyl end of the phosphorylatable amino acid must be an acidic residue [29]; aspartic or glutamic acid in this position can be replaced by phosphoserine [30].

In our previous report we mapped several functional protein kinase CK2 phosphorylation sites within the C-terminal region of CTCF, and demonstrated their importance for regulation of CTCF by CK2. In this report we further investigate the functional role of CK2 phosphorylation in the regulation of the activity of CTCF. We demonstrate that coexpression of CTCF with protein kinase CK2 change CTCF repressior function into an activator function. Mutating all CK2 phosphorylatable residues into non-phosphorylatable residues (phospho-ablation mutant) or into acidic residues (phospho-mimetic mutant) generated proteins that did not respond to the exogenous CK2. The phospho-ablation mutant increases the repression tendency of CTCF in the context of the c-myc promoters, whereas, the phospho-mimetic mutant of CTCF behaves as an activator. Serine-612 has been identified as a critical residue in the

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Abbreviations: CTCF, CCCTC binding transcription factor; CK2, protein kinase CK2; pCK2, catalytic α-subunit of protein kinase CK2; pmCK2, kinase inactive mutant of the catalytic α-subunit of protein kinase CK2; CAT, chloramphenicol acetyltransferase

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functional regulation of CTCF by phosphorylation and, finally, the phospho-ablation, but not phospho-mimetic, mutant of CTCF potentiated the growth-suppressive ability of the wild-type protein in COS 7 cells.

2. Materials and methods

2.1. Generation of the CTCF mutants deficient in CK2 phosphorylation

In order to investigate the role of CK2 in modification of CTCF, serines matching the consensus site of CK2 (S∗/T∗XXDE) within the C-terminal region of CTCF *CS(578)GLDRS(604)KKEDS(609)S(610)DS(612)EE…S(724)MMDR* were mutated to generate the non-phosphorylatable (phospho-ablation) CTCF and the acidic (phospho-mimetic) mutants. The two-step PCR-mediated mutagene procedure [31] was used to substitute serine residues with alanines/glycines or glutamic acid. The external rightward primer carrying the wild-type sequence was 5′-*ACTCAAAGT**GGTACC**ATGAAGATG-CAC-3′*, starting at nucleotide position 1707-bp of the CTCF cDNA sequence, and harbouring consensus site for *Kpn*I (underlined), numbered according to Klenova et al. [2]. The external leftward primer was *5′-TC**ACCGGT**CCATCATGCTGAGAATCAT-3′*, starting at nucleotide position 2652 and encompassing consensus site for *Age*I (underlined). For each mutant, two internal self-complementary (mutagenic) primers were utilised (sequences are shown in brackets in the description of the mutants below, mutated nucleotides are presented in bold).

The phospho-ablation CTCF mutants can be described as follows: pAla^{604,609,610,612} (substituted Ser-604, 609, 610 and 612; 5′(2261 bp) ATGCGC**GCT**AAGAAAGAAGAT**GCCGGC**GAT**GCT**GAGGAA/3′-TACGCG**CGA**TTCTTTCTTCTA**CGGCCG**CTA**CGA**CTCCTT); pAla^{578} (substituted Ser-578; 5′(2181 bp)-GATAACTGT**GCT**GGCC-TAGAT/3′-CTATTGACA**CGA**CCGGATCTA); pAla^{604} (substituted Ser-604; 5′(2255 bp)-AGGAAGATGCG**GCT**AAG/3′-TCCTTCTA-CGCG**CGA**TTC); pAla^{612} (substituted Ser-612; 5′(2289 bp)-GAT**GCT**GAGGAAAATGCTGAA/3′-CTA**CGA**CTCCTTTTACG-ACTT); pAla^{604,612} (substituted Ser-604 and 612; 5′(2261 bp)-ATGCGC**GCT**AAGAAAGAAGATTCCTCCGAT**GCT**GAGGAA/3′-TACGCG**CGA**TTCTTTCTTCTAAGCAGGCTA**CGA**CTCCTT); pAla^{578,604,609,610,612} (substituted Ser-578, 604, 609, 610 and 612) was generated with the primer described for pA1a578 using the mutant DNA of pAla^{604,609,610,612} as a template for amplification. Finally, the acidic (phospho-mimetic) mutant can be described as pGlu^{604,609,610,612} (substituted Ser-604, 609, 610 and 612; 5′(2261 bp)-ATGCGC**GAG**AAGAAAGAAGAT**GAAGAA**GAT**GAA**GAGGAA/3′-TACGCG**CTC**TTCTTTCTTCTA**CTTCTT**CTA**CTT**CTCCTT).

To make pAla^{724} mutant (Ser-724 substituted with Ala), one-step PCR was run with the rightward primer, starting at 1707-bp and carrying the wild-type sequence. The leftward primer carried mutated bases (5′(2651 bp)-ATGATTCTC**GCC**ATGATGG***ACCGGTGA***-TGGAGG/TACTAAGAG**CGG**TACTACCTGGCCACTACCTCC); and started at 2651-bp. The amplified products were purified, double digested with *Kpn*I and *Age*I, and recloned into *Kpn*I–*Age*I sites within backbone of the wild-type cDNA of CTCF expressing construct (pCTCF) described previously [15]. The mutated variants of CTCF were verified by sequencing.

2.2. Reporter and expression constructs

The expression construct pRC/CMV/(HA-CK2α) of the catalytic α-subunit of protein kinase CK2 (abbreviated here as pCK2) and kinase inactive CK2α K68M mutant (abbreviated here as pmCK2) were provided by D. Litchfield and previously described by Penner et al. [32]. The chicken c-myc promoter-based construct, pPst2CAT, contains the full-length promoter (3.2 kb) cloned upstream of the coding region of the CAT reporter gene, and harbours a known CTCF binding site, FpV [1]. The reporter plasmid, p90TKCAT, was constructed by ligating the dimeric CTCF binding site (synthetic 90 oligomer) from the chicken c-myc promoter (−219 to −180 bp) [(*Hin*dIII) 5′-AGCTT*GAGCCCCTCGGCCGCCCCCTCGCGGCGCGCCCTCCC-CGC*ACTAGT*GAGCCCCTCGGCCGCCCCCTCGCGGCGCGCCC-TCCCCGC*T-3′ (*Xba*I)] into the *Hin*dIII–*Xba*I site of the CAT vector, pGLCAT4 [33] and termed pminCAT. The *Hin*dIII and *Xba*I sites were created at the ends of the oligonucleotides to facilitate ligation into pGLCAT4. The FPV (monomer binding site) is shown in italic.

The pPst2(Nsi)CAT reporter carries a mutation in the CTCF-binding site [2]. A mutant version of the p90TKCAT deficient for CTCF binding (p90(Nsi)TKCAT), was made by ligating the mutant dimeric (synthetic 90-bp oligomer) fragment, FpV(Nsi) [AGCTTGAGCCC-CTCGGCCGCCCCCT***ATGCAT***GCGCCCTCCCCGCACTAGTG-AGCCCCTCGGCCGCCCCCT***ATGCAT***GCGCCCTCCCCGCT] into *Hin*dIII–*Xba*I sites of pGLCAT4 vector. The FPV (monomer binding site) is shown in italic and the *Nsi*I-mutation is shown in bold italic. The *Spe*I site separating the two monomer CTCF binding sequences is underlined.

The albumin and adenovirus major late promoter-based constructs, pAlb-CAT and pAd-MLP-CAT, respectively, were provided by Dr. Nerlov and described in Nerlov and Ziff [34]. The pGal4-CTCF is a full-length CTCF cDNA fused, in frame, to the cDNA of the DNA-binding domain of yeast transcription factor, Gal4, and the p(6× UAS)TKCAT bears six copies of the Gal4 binding sites (UAS) cloned upstream of pBLCAT2 reporter. There two vectors were kindly provided by R. Renkawitz and L. Burke and described in Lutz et al. [35].

2.3. Cell culture and transfection procedure

COS 7 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) fetal calf serum. DNA transfection was carried out by the standard calcium phosphate precipitation method [36]. To monitor transfection efficiency, 50 ng of the plasmid pRSV-β-Gal was used in all transfection experiments as an internal reporter control. Cells were harvested 48 h post-transfection and the activities of chloramphenicol acetyltransferase (CAT) and β-galactosidase were assayed as previously described. The CAT activities were then normalised to the activities of β-galactosidase.

2.4. Cell clonogenicity assay in COS7 cells

For stable transfection with CTCF mutants, the cells were plated (in duplicate) at 1.5×10^6 per 10 cm plates containing 10 ml of DMEM medium. Twenty-four hours post-plating, cells were cotransfected with 30 μg of expression constructs encoding CTCF mutants and 3 μg of geneticin-resistant plasmid, pSV2neo. Twenty-four hours post-transfection, the cells were trypsinised, and single cell suspension in fresh medium was prepared. Cells were then re-seeded onto new plates in different dilutions, 0.75×10^6, 0.45×10^6, 1.5×10^5 and 0.75×10^5 cells per plate. Forty-eight hours later, geneticin (Gibco-BRL), at final concentration of 1 mg/ml, was added to media for selection. The cells were maintained in selective medium for 18 days, during this time the selective medium was changed every 3 days. Selective medium contained 50% of fresh DMEM, 50% of the conditioned growth factor-enriched medium and geneticin (1 mg/ml).

Cell labeling and immunoprecipitation were performed as reported by Klenova et al. [15].

2.5. Western blotting

Proteins were resolved, transferred onto a membrane and probed as described previously [37]. The primary antibodies used in this study were the anti-CTCF polyclonal (Abcam) (1:300), the anti-α-CK2 polyclonal (gift from D. Litchfield) (1:1000) and the anti-α-tubulin monoclonal (Sigma) (1:500). The secondary anti-rabbit-peroxidase or anti-mouse-peroxidase conjugated antibodies (Dako) were used at 1:10 000 dilution. The antibodies were visualised by Enhanced Chemiluminescence as recommended by the supplier (Amersham).

3. Results

CK2 relieves the transcriptional repression of the chicken c-myc reporters mediated by CTCF. To investigate the functional consequence of the phosphorylation of CTCF by CK2 in vivo, the catalytic α-subunit of CK2- and the chicken CTCF-encoding constructs, pCK2 and pCTCF, respectively, were co-expressed in COS7 cells. The cells were transfected with 0.5 μg of either the chicken c-myc promoter-based CAT reporter, pPst2CAT, or its mutated version pPst2(Nsi)CAT, which is deficient for CTCF binding, along with 1.0 μg of pCTCF and increasing concentrations (1.0 and 2.0 μg) of

pCK2 (Fig. 1A). Efficient expression of the α-subunit of CK2 was reported previously [32] and was confirmed in our experiments (Fig. 1D). No change in the levels of CTCF, endogenous and exogenous, was noted upon the increasing expression of CK2 (Figs. 1D and 2B). Nevertheless, as shown in Fig. 1E, the exogenously supplied CK2 was able to efficiently phosphorylate CTCF.

Cotransfection of both reporters with the empty vector, pSG5, resulted in comparable transcriptional activities. In the absence of CK2-encoding construct, the exogenous CTCF inhibited expression of the wild-type reporter. However, 1.0 μg of the pCK2 in the transfection mixture relieved this construct from inhibition by the exogenous CTCF, and the increasing dose of pCK2 progressively stimulated the activity of the

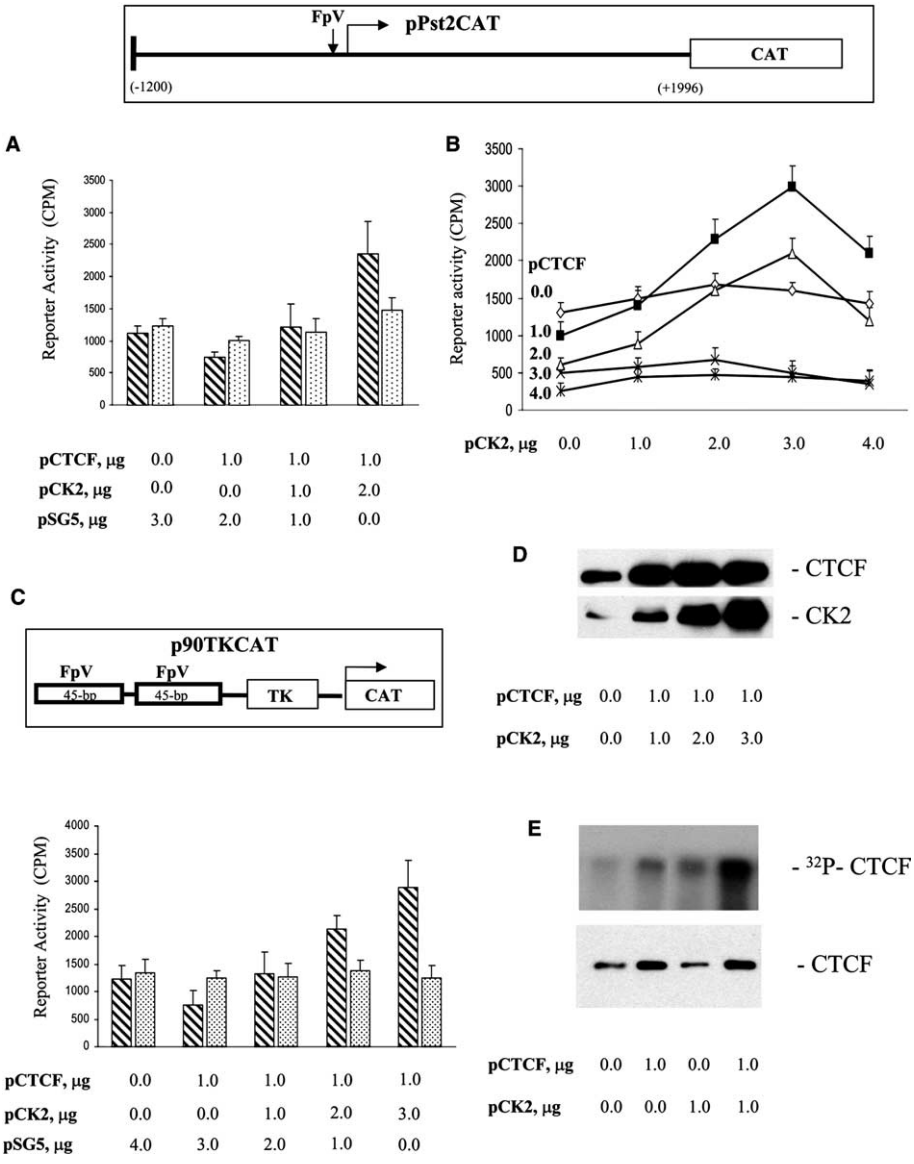

Fig. 1. CK2 relieves CTCF-dependent transcriptional repression activity of the chicken c-myc promoter and the minimal chicken c-myc promoter. COS7 cells were transfected with 0.5 μg of the chicken c-myc reporter, pPst2CAT (bars with striped filling) or its mutant version, pPst2(Nsi)CAT (bars with dotted filling) (A) or with 0.5 μg of the p90TKCAT (bars with striped filling) or its mutant version, p90(Nsi)TKCAT (bars with dotted filling) (C), together with the indicated concentrations (μg) of the shown constructs. Schematic map (not to scale) of the wild-type reporters are shown on top of the figures. The results shown represent the mean values with the standard deviations (error bars) of triplicates in one typical cotransfection experiment. The experiment was repeated five times. (B) Cotransfection experiments of 0.5 μg of the chicken pPst2CAT reporter along with the increasing concentrations of pCTCF and pCK2. DNA was balanced in all transfections with the parent expression vector, pSG5. Forty-eight hours post-transfection, total cell lysates were prepared, assayed for CAT activity and normalised to β-Gal activity as described in Section 2. (D) Ectopic CK2 is efficiently expressed in COS7 cells and CTCF levels do not alter upon CK overexpression: Western immunoblotting with anti-CTCF (top) and anti-αCK2 (bottom) antibodies of the samples from transiently cotransfected COS7 cells. Membrane was first probed with anti-CTCF antibody, stripped and then probed with anti-αCK2 antibody. Numbers below show the amount of the transfected vectors. Positions of CTCF and αCK2 are shown. (E) Cotransfection with pCK2 leads to CTCF phosphorylation in vivo. COS7 cells were cotransfected with the amount of pCTCF and pCK2 as indicated in the Figure. Cells were then metabolically labelled with [^{32}P]orthophosphate, and CTCF was immunoprecipitated from the lysates as previously described [15]. Protein samples were then resolved in 10% SDS–PAGE, blotted onto a membrane and exposed to an X-ray film (top). The same membrane was subsequently probed with the anti-CTCF antibodies (bottom). The position of CTCF is indicated.

promoter. As observed previously [3,15], there was also an increase in CAT expression from the mutant reporter, but much less pronounced than that shown by the wild-type construct.

We then compared the expression patterns of pPst2CAT induced by different combinations of pCK2 and pCTCF concentrations. As the data in Fig. 1B show, in the context of the chicken reporter, pPst2CAT, the effects from pCK2 were more pronounced with 1.0 and 2.0 μg of the pCTCF. The general trend of the reporter namely, upregulation, with these doses was essentially similar. Thus, 3.0 μg of pCK2 enhanced CAT expression to 2.5- and 1.5-fold, respectively, over its basal level. Nonetheless, at 4.0 μg of the pCK2 transcription from pPst2CAT was decreased, which could be an indirect effect (e.g., squelching) mediated by the pCK2. At higher doses of pCTCF (3.0 and 4.0 μg), the plateau of repression emerged, where the increasing doses of pCK2 did not relieve inhibition by pCTCF.

To narrow down functional contribution of possible direct or indirect activities of other transcription factors binding to the pPst2CAT, the effects of CK2 on CTCF were tested in the context of the minimal promoter-driving reporter genes, p90TKCAT and its mutated version, p90(Nsi)TKCAT. As shown in Fig. 1C our observations using the natural c-myc promoter-based reporter (pPst2CAT) were confirmed in the context of the minimal wild-type reporter, p90TKCAT. This indicates that the increment of the expressed CAT level, from both natural and minimal c-myc promoter-based reporters, is very likely due to the specific effect of CK2 on the CTCF. Inability of pCK2 to stimulate transcriptional activity of the mutant reporter, p90(Nsi)TKCAT, indicates that effects of the CK2 on the CTCF are dependent on the CTCF binding to its cognate site.

Taken collectively, these experiments show that CK2 switches transcriptional inhibitory function of CTCF into an activating mode, ultimately leading to the increase of basal transcriptional activity from the chicken c-myc promoter.

CK2 relieves transcriptional repression activity of the chimeric promoter-based reporter, p(6 × UAS)TKCAT, mediated by Gal4-CTCF. To test that CK2 effects on CTCF are direct in the context of the designed promoter, a composite promoter-based reporter, p(6 × UAS)TKCAT, and the hybrid, pGal4-CTCF, were utilised. In these experiments, COS7 cells were cotransfected with 0.5 μg of p(6 × UAS)TKCAT reporter together with 1.0, 2.0 and 3.0 μg of the pGal4-CTCF. As shown in the Fig. 2A, the recruitment of CTCF into the vicinity of transcriptional machinery of the TK promoter inhibits, in a dose-dependent manner, the basal transcription of the reporter gene. However, the introduction of pCK2 in a transfection mixture converted repression tendency of pGal4-CTCF into an activation mode, in a dose-dependent manner. Western blot analysis confirmed that this activation was indeed accompanied by the increasing levels of CK2 (Fig. 2B). Lack of any activity from the control assay (co-expression of the reporter together with pGal424 and pCK2) supports the view that all these activities are likely to be specific effects of CK2 on the CTCF protein.

Since there is no known mammalian protein with the same consensus DNA-binding site as Gal4 [38], this assay is likely to reflect the specific role of CK2 on CTCF, in the situation when there is no interference with the endogenous CTCF protein. This is important since binding of the endogenous CTCF phosphorylated by CK2 to its site in other natural (e.g., pPst2CAT) or chimeric (e.g., p90TKCAT) promoter-based construct may to some extent obscure the effects of CK2 on the ectopic CTCF.

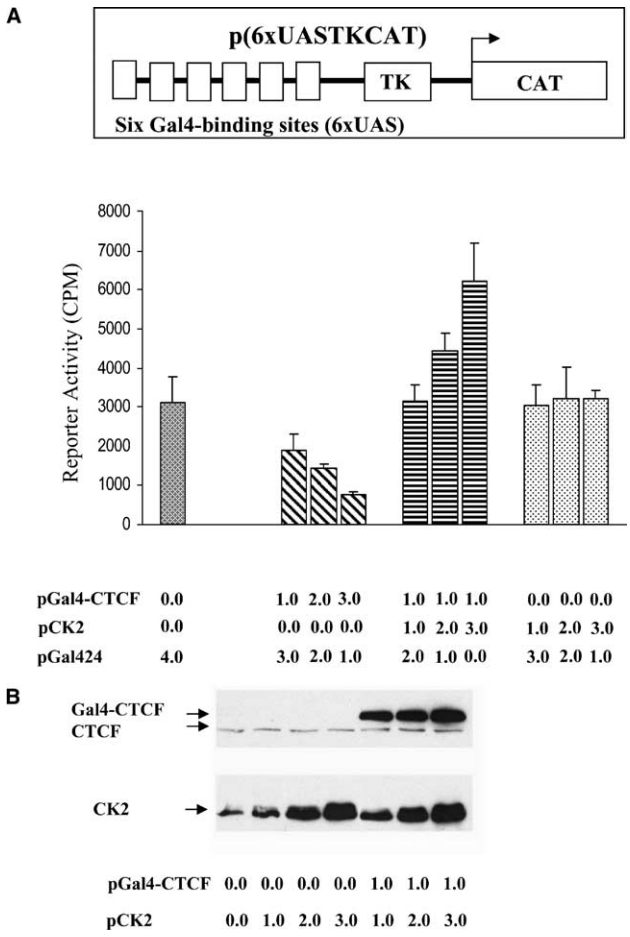

Fig. 2. CK2 relieves transcriptional repression of the minimal promoter-based reporter, 6 × UAS(TKCAT), mediated by the heterologous protein, Gal4-CTCF. **(A)** COS7 cells were transfected with 0.5 μg of the p(6 × UAS)TKCAT along with the indicated concentrations (μg) of the shown vectors. Schematic map (not to scale) of the wild-type reporters are shown on top of the figure. Forty-eight hours post-transfection, total cell lysates were prepared, assayed for CAT activity and normalised to β-Gal activity as described in Section 2. The results shown represent the mean values with the standard deviations (error bars) of triplicates in one typical cotransfection experiment. The experiment was repeated seven times. (B) Ectopic CK2 is efficiently expressed in this experiment and levels of Gal4-CTCF and the endogenous CTCF do not alter upon CK overexpression: Western immunoblotting with anti-CTCF (top) and anti-αCK2 (bottom) antibodies of the samples from transiently cotransfected COS7 cells. Membrane was first probed with anti-CTCF antibody, stripped and then probed with anti-αCK2 antibody. Positions of Gal4-CTCF, endogenous CTCF and CK2 are indicated. Numbers below show the amount of the transfected vectors.

The kinase inactive mutant, pmCK2, does not relieve transcriptional repression mediated by CTCF. We next asked if the effects observed in our cotransfection experiments were indeed dependent on functional CK2. To address this question we employed the kinase inactive CK2alpha K68M mutant (pmCK2) in cotransfection experiments with pCTCF and reporters p90TKCAT and p(6xUAS)TKCAT. As shown in Figs. 3A and C, no effects from pmCK2 were detected with either reporter construct, although levels of expression from

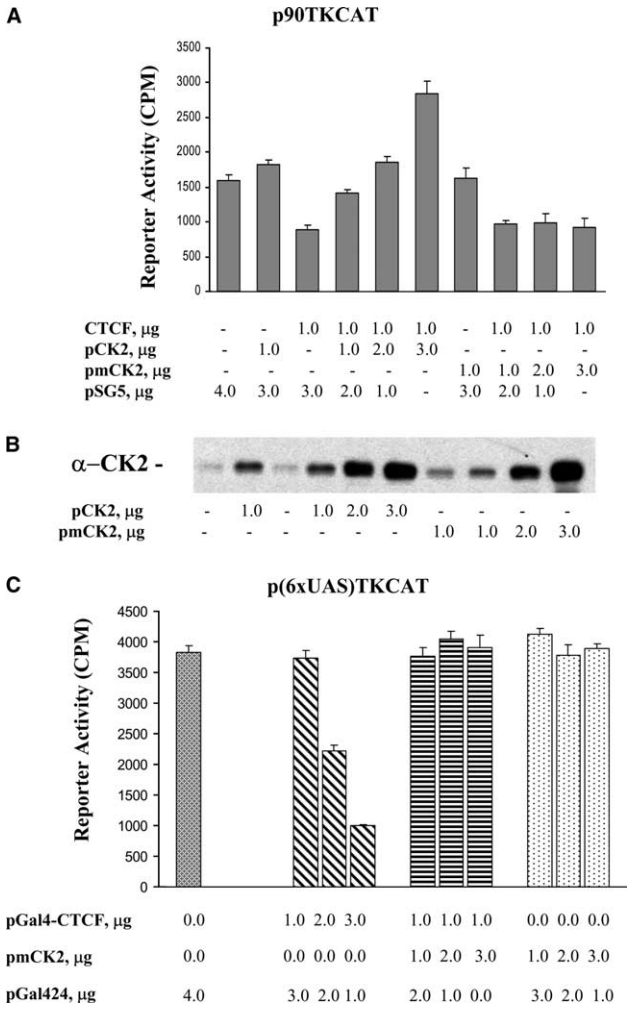

Fig. 3. The kinase inactive mutant does not relieve transcriptional repression mediated by CTCF. COS7 cells were transfected with 0.5 μg of the p90TKCAT (A) or p(6xUAS)TKCAT (C) along with the indicated concentrations (μg) of the shown vectors. Forty-eight hours post-transfection, total cell lysates were prepared, assayed for CAT activity and normalised to β-Gal activity as described in Section 2. The results shown represent the mean values with the standard deviations (error bars) of triplicates in one typical cotransfection experiment. The experiment was repeated five times. (B) Levels of the expression of CK2 and the kinase inactive mutant of CK2 correlate with the input of the corresponding plasmids as shown by Western immunoblotting with the anti-αCK2 antibodies of the samples from transiently cotransfected COS7 cells in the experiment described in (A). Position of CK2 is indicated. Numbers below show the amount of the transfected vectors.

pmCK2 were comparable with the levels from pCK2 in these series of experiments (Fig. 3B). Taking together, all the above data demonstrate that phosphorylation of CTCF by CK2 plays an important role in regulating transcriptional activity of the CTCF protein.

The phospho-ablation and phospho-mimetic mutants of CTCF recapitulate transcriptional activities of the endogenous hypo- and hyper-phosphorylated CTCF proteins, respectively, in the context of the c-myc promoter constructs. Having established that protein kinase CK2 relieved promoters from inhibition by CTCF, we extended this line of research to gain further evidence that all these activities are mediated by direct effects of CK2 on CTCF. For this purpose, the expression constructs encoding a series of mutated CTCF derivatives at the CK2 phosphorylatable residues were made. The phospho-ablation and phospho-mimetic CTCF mutants were generated by replacing potential CK2 phosphorylatable serine residues in the C-terminal region with alanine/glycine and glutamic acid, respectively (see Section 2 for detail). These mutant constructs were based on the fact that substitution of the CK2 serines, 604, 609, 610 and 612, by non-phosphorylatable residues completely abolished the ability of CTCF to be phosphorylated in vivo [15]. Two other residues, Ser-578 and Ser-724, were also replaced by alanine residues. Although neither of these serines were not shown to be phosphorylated in vivo in COS7 cells [15], they are located within consensus motif of CK2 [22]. We reasoned that these sites might still be functional in other cellular contexts and the individual mutants carrying substitutions of these serines with alanine residues were also produced. Some of the CTCF mutants harboured one substituted serine residue, while others included different combinations of the replacements. Of note, substitution of serines by alanines/glycine or glutamic acid did not alter localisation of these mutant isoforms since all of them could be detected in the nucleus of COS7 cells after transfection ([15] and data not shown).

The transcriptional properties of the CTCF mutants were tested in the context of the chicken c-myc promoter-based reporter construct, pPst2CAT, and the results are summarised in Fig. 4A. Expression constructs of the wild type and of the mutants were cotransfected into COS7 cells in the increasing doses (1.0, 2.0 and 3.0 μg). Levels of expression of the mutated variants were similar to the levels of the wild-type protein in the transfected cells and proteins were produced according to concentrations of the corresponding expressing constructs (Fig. 4B).

In agreement with our previous observations [15], the CK2 site-deficient mutant, $pAla^{604,609,610,612}$, exerted a stronger inhibitory influence than the wild-type CTCF protein-encoding construct (pCTCF) at all three concentrations of the transfected expressing vectors. Thus, while 1.0 μg of the wild-type pCTCF reduced CAT level down to 75% of its basal activity, this mutant repressed it down to 30% of the basal activity. Addition of 2.0 μg of each expressing plasmid resulted in further transcription repression down to 60% by the wild-type protein and down to 25% by the $pAla^{604,609,610,612}$; 3.0 μg of the expressing constructs of pCTCF and $pAla^{604,609,610,612}$ brought further reduction of the CAT level, which was much more pronounced for the mutant protein.

Substitution of individual serines with alanines led to differential response from the reporter, which ranged from repression to activation. Thus, single mutations of serines 578 ($pAla^{578}$) and 604 ($pAla^{604}$) created mutants that showed repressor ability similar to the wild-type protein. On the other hand, knocking out Ser-612 generated a repressor ($pAla^{612}$) that had a stronger inhibitory effect on the reporter than the wild-type protein. Mutation of two serines 604 and 612 ($pAla^{604,612}$) did not potentiate, in additive manner, inhibitory function of the single mutant, $pAla^{612}$. Strikingly, one mutant, $pAla^{724}$, increased the constitutive activity of the reporter in a dose-dependent manner. Substitution of five serines in the C-terminal region led to a potent mutant ($pAla^{578,604,609,610,612}$), which inhibited expression of the reporter protein down to 25% of its basal level. On the other hand, the acidic mutant of CTCF, $pGlu^{604,609,610,612}$, behaved as a transcriptional activator in this assay. Although lower dose (1.0 μg) of this construct did not promote basal CAT level, the highest dose

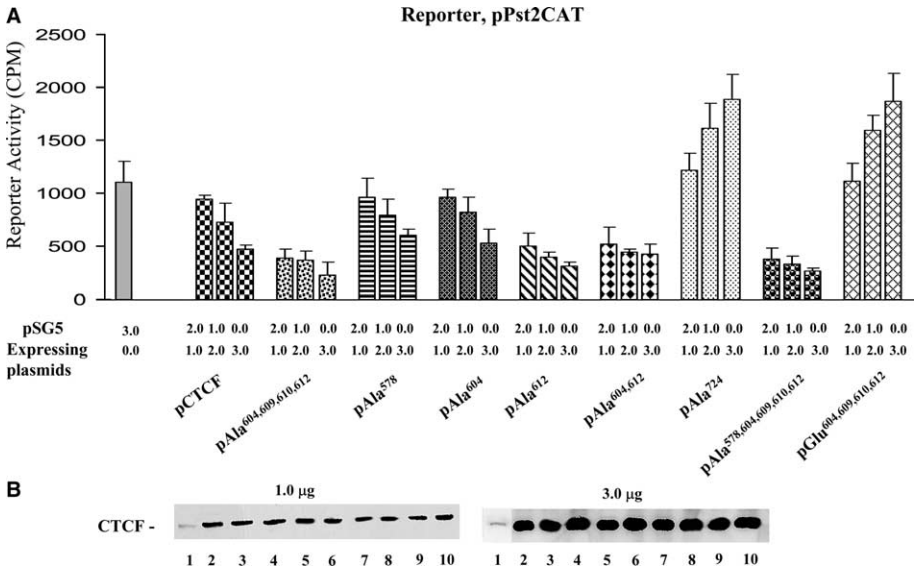

Fig. 4. The CK2 site-deficient mutants of CTCF have distinct mode of action in the context of the chicken c-myc promoter. (A) COS7 cells were transfected with 0.5 μg of the chicken c-myc reporter, pPst2CAT, along with the reported amounts (μg) of the shown constructs. Total DNA concentrations were balanced in all experiments with the parent expression vector, pSG5. Forty-eight hours post-transfection, total cell lysates were prepared, assayed for CAT activity and normalised to β-Gal activity as described in Section 2. The results shown represent the mean values with the standard deviations (error bars) of triplicates in one typical cotransfection experiment. The experiment was repeated five times. (B) Western blot showing levels of the CTCF proteins generated from the 1 (left) and 3 (right) μg of the following constructs: (1) pSG5, (2) pCTCF, (3) $pAla^{604,609,610,612}$, (4) $pAla^{578}$, (5) $pAla^{604}$, (6) $pAla^{612}$, (7) $pAla^{604,612}$, (8) $pAla^{724}$, (9) $Ala^{578,604,609,610,612}$ and (10) $pGlu^{604,609,610,612}$.

(3.0 μg) stimulated CAT expression 45% over its basal level. Thus, the presence of the constitutive negative charge at the C-terminal region of CTCF obviated requirement for CK2.

Similar observations were made when a different reporter, p90TKCAT, was used in these experiments: each mutant recapitulated its specific mode, which was observed in the context of the natural c-myc promoter-based reporter (data not shown). From these data we concluded that: (1) Ser-612, appears to play the key role in the regulation of the chicken c-myc promoter. (2) The four-site ($pAla^{604,609,610,612}$) and five-site ($pAla^{578,604,609,610,612}$) mutants have pronounced transcriptional repression functions, which may result from the cumulative effect of single serines. (3) The data showing that knocking out Ser-724 created the CTCF protein with features of an activator indicate that complex combinatorial effects of the phosphoacceptor sites regulate CTCF transcription properties.

Transcriptional behaviour of CTCF mutants is specific to CTCF-dependent promoters. This line of research was continued to test transcriptional properties of the CTCF mutants in the contexts of other promoters, not known to be CTCF-responsive targets. In these experiments, cotransfection of 1.0, 2.0 and 3.0 μg of each of the wild type, $pAla^{604,609,610,612}$ and $pGlu^{604,609,610,612}$ along with pAlb-CAT, the albumin promoter-driving CAT, did not alter basal CAT level of this reporter construct. As shown in Fig. 5B, proteins were produced according to concentrations of the corresponding expressing constructs. Similar results were obtained when an adenovirus-major late gene promoter fused to the CAT gene, pAdMLP-CAT, was used as a reporter in cotransfection experiments (data not shown). Taken together, these observations suggest that effects of the CK2 site-deficient mutants of CTCF are specific to the CTCF-dependent promoters, and that replacing Ser with Ala/Gly residues did not create fortuitously a constitutive transcriptional repressor.

Exogenous CK2 does not relieve repression of the chicken c-myc reporter constructs induced by the CTCF mutants, $pAla^{604,609,610,612}$, $pAla^{578,604,609,610,612}$, $pAla^{612}$ and $pAla^{604,612}$. To test the effects of the ectopic CK2 on the specific behaviour of the phopshorylation deficient CTCF variants in the context of the chicken c-myc promoter, COS7 cells were transfected with 1.0 μg of each of CTCF mutants along with the increasing concentrations (0.0, 1.0, 2.0, 3.0 and 5.0 μg) of pCK2. The equivalent expression of the proteins from each expressing construct was confirmed by Western analysis (Fig. 6B). As shown in Fig. 6A, the wild-type construct, pCTCF, exhibited a typical dose-dependent response to pCK2. On the other hand, non-phosphorylatable mutants, which imposed stronger inhibitory role than that of the wild-type protein on the reporter (Fig. 4A), resisted stimulating action of the pCK2. This can be demonstrated with the $pAla^{604,609,610,612}$ and $pAla^{578,604,609,610,612}$ mutants. On the other hand, the single mutants such as $pAla^{578}$ and $pAla^{604}$, which exhibited wild-type protein-inhibitory ability (Fig. 4A), showed positive response to pCK2 comparable to the wild-type vector. The single ($pAla^{612}$) and double ($pAla^{604,612}$) mutants did not show any significant response to CK2. However, the highest dose of the pCK2 (5.0 μg) had positive effect, which could be due to indirect effect of the kinase. Strikingly, one mutant, $pAla^{724}$, exhibited negative response to the pCK2, in a dose-dependent manner. Finally, the acidic mutant, $pGlu^{604,609,610,612}$, also resisted the effect of pCK2.

The effects of CK2 on the mutant proteins observed in the context of the natural c-myc promoter, pPst2CAT were recapitulated in the context of the p90TKCAT minimal promoter (data not shown).

Taking all these data collectively, we conclude that (1) the CK2 did not relieve inhibitory effect of $pAla^{604,609,610,612}$, $pAla^{612}$, $pAla^{604,612}$ and $pAla^{578,604,609,610,612}$, in the contexts

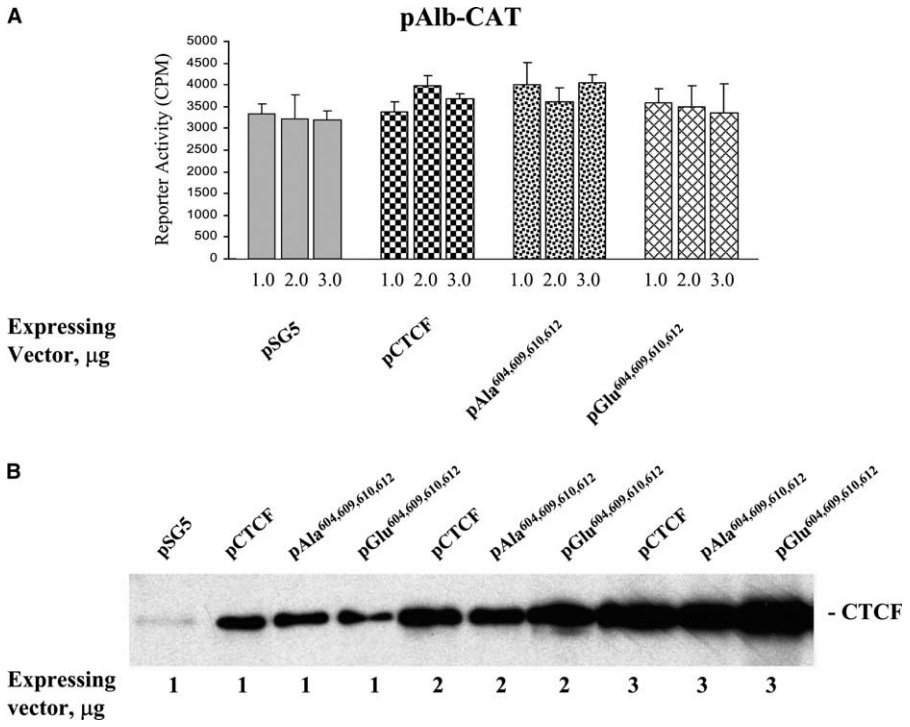

Fig. 5. The wild-type phospho-ablation and phospho-mimetic mutants of CTCF do not alter the basal transcription activity of the albumin (Alb) promoter-based reporter. (A) COS7 cells were transfected with 0.5 μg of the albumin pAlb-CAT promoter-based vector along with reported concentrations (μg) of the wild type, and phopsho-ablation ($pAla^{604,609,610,612}$) and phospho-mimetic ($pGlu^{604,609,610,612}$) mutants of the CTCF and the empty vector, pSG5. Forty-eight hours post-transfection, total cell lysates were prepared, assayed for CAT activity and normalised to β-Gal activity as described in Section 2. The results shown represent the mean values with the standard deviations (error bars) of triplicates in one typical cotransfection experiment. The experiment was repeated five times. (B) Western blot of the samples from this experiment shows the levels of the CTCF proteins generated from the 1, 2 and 3 μg of the wild-type CTCF and mutant constructs. The position of CTCF is indicated.

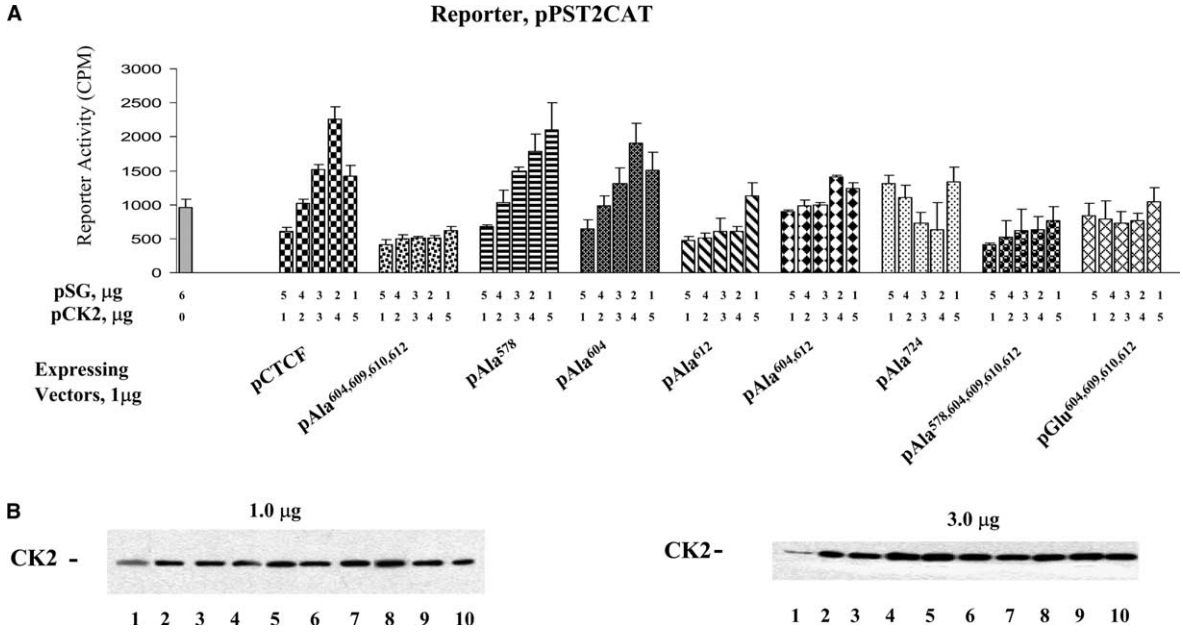

Fig. 6. Effects of CK2 phosphorylation-deficient mutants of CTCF cotransfected with CK2 on the chicken c-myc promoter constructs. (A) COS7 cells were transfected with 0.5 μg of the chicken natural c-myc promoter-based reporter, pPst2CAT, 1.0 μg of the reported CTCF expressing vectors together with 0.0, 1.0, 2.0, 3.0 and 5.0 μg, respectively, of CK2-expressing construct. Total DNA concentration was balanced in all experiments with the parent expression vector, pSG5. Forty-eight hours post-transfection, total cell lysates were prepared, assayed for CAT activity and normalised to β-Gal activity as described in Section 2. The results shown represent the mean values with the standard deviations (error bars) of triplicates in one typical cotransfection experiment. The experiment was repeated five times. (B) Western blot showing levels of the CK2 protein generated from the 1 (left) and 3 (right) μg after co-transfection with 1 μg the following constructs: (1) pSG5, (2) pCTCF, (3) $pAla^{604,609,610,612}$, (4) $pAla^{578}$, (5) $pAla^{604}$, (6) $pAla^{612}$, (7) $pAla^{604,612}$, (8) $pAla^{724}$, (9) $Ala^{578,604,609,610,612}$ and (10) $pGlu^{604,609,610,612}$.

of the chicken c-myc reporters and (2) the exogenous CK2 was able to activate pAla^{578} and pAla^{604} mutants.

The phopsho-ablation mutant of CTCF suppresses cell growth of COS7 cells more efficiently than the wild-type CTCF. Ectopic expression of CTCF in many cell types inhibited cell clonogenicity by causing profound growth retardation [39]. In this report, we demonstrated that dephosphorylated CTCF mutant (pAla^{604,609,610,612}) exerted stronger inhibitory function than the wild-type protein on the c-myc promoter. As the c-myc gene is one of the prominent proliferation-controlling genes, we then asked if the substitution of the serine residues in the C-terminus of CTCF would interfere with the ability of CTCF to suppress the proliferative potential of COS7 cells.

For this purpose, COS7 cells were stably transfected with the plasmids expressing individual CTCF mutants and the vector pSV40neo containing a neomycin resistance gene. At the 10:1 ratio between these plasmids all the neomycin resistant cells were presumed to contain the CTCF-expressing plasmid. A control pSG5 plasmid that does not express CTCF was also exploited to establish the basal focus-forming potential of cells in the absence of any exogenous CTCF isoform. Twenty-four hours post-transfection, cells were subjected to G418 selection and two weeks later the number of colonies was counted. Ten independent colonies from each transfection were picked, mixed and expanded. The cells were then collected, lysed and the Western assay performed. As shown in Fig. 7B, the levels of the exogenous CTCF (wild type and mutated) expressed in these cells are comparable.

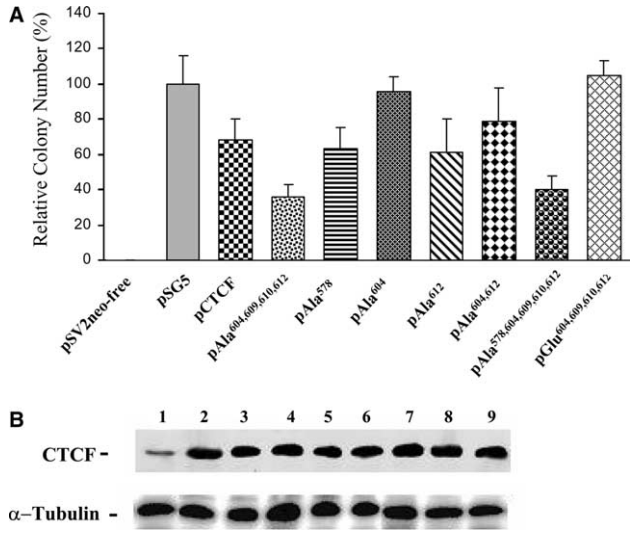

Fig. 7. Effects of mutations of the CK2 phosphorylation sites on the ability of CTCF to suppress growth of COS7 cells. (A) COS7 cells were stably cotransfected with 3.0 with the indicated expression constructs and selected with G418 as described in Section 2. In some plates (pSV2neo-free), cells were cotransfected with pSG5 without the G418 resistant plasmid, pSV2neo. The 100% value was assigned to the numbers of colonies formed with the pSG5 control vector. The error bars indicate standard deviation of four independent transfections with each expression vector. (B) Western blot showing levels of the CTCF proteins generated from the lysates of 10 individual colonies collected and expanded from the stable transfection experiments with the following nine vectors: (1) pSG5, (2) pCTCF, (3) pAla^{604,609,610,612}, (4) pAla^{578}, (5) pAla^{604}, (6) pAla^{612}, (7) pAla^{604,612}, (8) Ala^{578,604,609,610,612} and (9) pGlu^{604,609,610,612}. 20 μg of the total protein was used in this experiment. Equal loading was confirmed by re-probing the membrane with the anti-α-tubulin antibodies. Positions of CTCF and α-tubulin are indicated.

The results of multiple assays for each mutant of CTCF are summarised in Fig. 7A. Colony formation of the transfectants was dependent in the presence of the pSV2neo plasmid since there was no cell growth in the absence of pSV2neo plasmid. The wild-type CTCF suppressed the number of growing foci down to 67% (the control vector pSG5 is taken as 100%). The substitution of alanines/glycines for any single serine did not significantly increase suppression efficiency of CTCF. Substitution of four or five serines, however, decreased the number of colonies to 35% for pAla^{604,609,610,612} and 40% for pAla^{578,604,609,610,612}. The acidic mutant of CTCF, pGlu^{604,609,610,612} exhibited the opposite effect, relieving growth suppression activity of the wild-type CTCF. Thus, the effects of only the phospho-ablation and phospho-mimetic CTCF mutants in the colony-formation assay paralleled their functional roles in the context of c-myc promoter-based reporters.

4. Discussion

This work extends our previous study on regulation of a transcription factor, CTCF, by phosphorylation with protein kinase CK2 [15] providing further evidence about the functional significance of this post-translational modification of CTCF. We demonstrated that cotransfection of CTCF and CK2 switched the function of CTCF, in the context of the chicken c-myc promoter, from a transcriptional repressor to an activator. This transition depends on modification of CTCF by CK2, because (i) both natural and chimeric chicken c-myc promoter-based constructs exhibited progressive activation, which was dependent on the levels of CK2 and was more pronounced in the presence of the exogenous CTCF; (ii) these effects were CTCF-target site dependent since co-expression of pCTCF and pCK2 together with the reporter constructs bearing the CTCF-mutated site [pPst2(Nsi)CAT and p90(Nsi)TK-CAT] insignificantly activated basal expression of the reporters, (iii) the kinase-inactive mutant of CK2 had no effect in these assays and, finally, (iv) repression by the Gal-4-CTCF protein from the composite promoter-based reporter, p(6 × UAS)-TKCAT was found to be efficiently relieved by CK2. In addition, selective inhibitors of CK2 such as apigenin [40] and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) [41] abolished the activating influence of CK2 (E. Klenova, personal communication).

Studies on the CTCF mutants provided a further link between modification of CTCF by CK2 and activation of the chicken c-myc reporter constructs. It was previously shown that phosphorylation residues in CTCF are restricted to the motif spanning Ser-604 to −612 (18). In the contexts of all the c-myc promoter-based constructs, substitution of all serines, 604, 609, 610 and 612, with Ala created the CTCF isoforms (e.g., pAla^{604,609,610,612} and pAla^{578,604,609,610,612}) that exerted stronger inhibitory effects than the wild-type protein. This observation supports the model that activation of c-myc promoters induced by synergistic action of CTCF and CK2 is directly mediated by phosphorylation of CTCF at Ser-604/609/610/612. Furthermore, the pAla^{604,609,610,612}, pAla^{578,604,609,610,612} and pGlu^{604,609,610,612} mutants consistently resisted the effects of pCK2 in the contexts of all c-myc reporters, which implies that CK2 modifies the wild-type protein at those residues in vivo. The pAla^{604} and pAla^{578} had

wild-type protein-like response to CK2 confirming that Serine-604 and Serine-578 are not critical for CTCF function. Although the panel of CK2 phosphorylation deficient mutants, which was generated and tested in this report is not comprehensive, we provide evidence that Serine-612 has a critical role in regulation of the transcriptional function of CTCF because $pAla^{612}$ had functional abilities comparable with the non-phopshorylatable $pAla^{604,609,610,612}$ mutant. The importance of Ser-612 might have been attributed to its role as a ''gate-keeper'' in controlling phosphorylation of other upstream residues (e.g., Serines 604, 609 and 610). Further tests revealed that phopshorylation of CTCF does not occur when Ser-612 is mutated (data not shown) and this mutant was resistant to the ectopic CK2 (Fig. 6). It is conceivable that phosphorylation at this site may be sufficient to induce a particular conformation that is critical for the assembly of the functional protein associations involved in control of cellular functions. As known from the literature, phosphorylation of BAD at a single Ser-136 promotes association with 14-3-3 proteins [42] which results in its sequestering from the apoptotic machinery.

The substitution of Ser-724 into Alanine generated a CTCF mutant ($pAla^{724}$) that activated chicken c-myc promoter in COS7 cells. This result was surprising since Ser-724 was not found to be phosphorylated in COS7 cells in vivo [15]. The trivial explanation may be that this mutation generated a fortuitous protein with structural changes possibly resembling the phospho-mimetic mutant of CTCF. It is however possible that association with other proteins (e.g., Yb-1 or B23 [37,43] and others) or even with another molecule of CTCF [44] could protect Ser-724 from phosphorylation. Similar situations have been described in the literature. For example, p67 interaction with eIF2α protects the latter from phosphorylation [45]; likewise, association of replication factor C with PCNA conceals the phosphorylation sites on the PCNA molecule [46]. Another explanation for such discrepancy could be that the phosphate at the Ser-724 is particularly efficiently removed by serine/threonine protein phosphatases, for example PP2A [47].

The introduction of the negative charge at the phosphorylation sites by replacement of the serines (604, 609, 610 and 612) with the glutamic acid residues created a phopsho-mimetic CTCF mutant that stimulated all c-myc reporters (particularly at higher doses). This observation demonstrates that the presence of the constitutive negative charge carried by this mutant compensated requirement for CK2. Nonetheless, the inherent transcriptional activating role of this acidic mutant is weaker than that mediated by synergistic action of the wild-type protein and CK2. This could be due to the weak negative charge of the carboxylate group of the glutamic acid, which can not exclusively emulate functional role of phosphate moiety in the phosphorylated CTCF isoform. However, overexpression of this ''weak'' acidic mutant at higher levels (3.0 μg) compensated for the partial weakness of its charge. Thus, the ability of CTCF to change the mode of action depending on its phosphorylation status may shed light into its ability to function as a repressor or activator depending on cellular and promoter context [4,5].

In this study we also tested whether the substitutions of the CK2 sites could affect cell growth inhibition by CTCF. The obtained results show that the growth suppressive effects of the wild-type CTCF in COS7 were significant, but not very strong. Such a response may depend on cell type as our previously reported observations showed [39]. After transfection with the wild-type CTCF, HeLa, K562 and Raji cells demonstrated a reduction of colonies to 30–40% compared to 5–10% in other cell lines (PC3, 293 and HD3) [15,39]. In this report we observed that the substitution of four and five serines in $pAla^{604,609,610,612}$ and $pAla^{578,604,609,610,612}$ caused remarkable growth inhibition (Fig. 7). These results contradict our previous finding showing the release from repression in HD3 and PC3 cells by the phospho-ablation mutant [15]. It should however be noted that the CK2 phosphorylation sites were initially mapped in COS7 cells and therefore the function of CK2 phosphorylation may be reflected more accurately in the context of COS7 cells. Cellular context indeed may be a significant factor in determining the mode of action of a protein since different kinases and interacting proteins may be involved in regulation of its function as, for example, is though to be the case for the retinoblastoma tumor suppressor protein, pRb [48].

Although the effects of the mutants carrying single or double substitution of serines on the c-myc promoter were apparent, these mutants failed to alter significantly the cell growth-suppression abilities of the wild type CTCF protein. This is however not surprising because CTCF controls transcriptional activity of many genes involved in the regulation of cell proliferation including $p19^{ARF}$, polo-like kinase (PLK), Igf2 and others [4,5,19,49]. Thus, suppression or activation of cell proliferation may require a threshold of positive or negative charges carried by CTCF to have an impact on various genes regulated by CTCF, which may not be achieved at the level of single mutation. Cumulative effects mediated by mutating several serines have been also observed by other proteins, for example p53 protein. Single substitution of serine-9, 17 or 37 with alanine in p53 N-terminus did not alter growth-suppression ability of the wild-type p53, however, altering all serines (9, 17 and 37) to alanines produced remarkable effect on cell growth [50,51].

Thus, our observations provide further evidence that the phosphorylation status of CTCF has critical consequences on cell physiology, where either arrest of cell growth or induction of cell proliferation is required; deregulated phosphorylation levels of CTCF may uncouple these biological activities in one way or another. The intriguing question in this model is how the activation and repression of CTCF can be modulated by CK2 phosphorylation if this enzyme is known to be constitutively active [52]? Indeed this is a general question that relates to more than 160 substrates of CK2 [53]. The current understanding of CK2 regulation is that its activity may be controlled by mechanisms that do not involve receptors and second messengers, but rely on the change in CK2 localisation in the cell, modulation of CK2 levels, associations with protein partners, activity of phosphatases, effects of substrate and others ([53] and references therein). CTCF, as one of the substrates of CK2, may be a subject of such regulation, when phosphorylation marks are established by default; removal of these marks on the other hand may be a part of more controlled processes.

The final biological outcomes of impaired phosphorylation levels of CTCF, including loss or gain of phosphorylation site(s), may therefore trigger apoptosis or initiate uncontrolled cellular growth and oncogenic activation. It is particularly important in the view of the growing body of recent evidence demonstrating the role of CK2 in proliferation, cell survival and apoptosis ([22] and references therein). Given that CK2

levels are often elevated in cancers [22] it is tempting to speculate that overexpression of CK2 in cancer cells could lead to excessive phosphorylation of CTCF, which may result in non-controllable overproduction of c-myc and thus may be one of the mechanisms of maintenance and evolution of tumour cell population.

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